

Inverse Regulation of Hepatic α_{1B} - and β_2 -Adrenergic Receptors

Cellular Mechanisms and Physiological Implications

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Catecholamines control a wide variety of metabolic processes in the liver, including key steps in carbohydrate, lipid, and amino acid metabolism. Of the multiple subtypes of adrenergic receptors (AR) that have been identified by pharmacological means as well as by molecular cloning, two subtypes have major roles in the control of hepatic functions in the rat: calcium-linked α_{1B} AR and cAMP-linked β_2 AR. The most extensively studied metabolic effect of catecholamines in the liver is glycogenolysis and the subsequent release of glucose, brought about by activation of the rate-limiting enzyme, glycogen phosphorylase. Phosphorylase activation by catecholamines can occur both through α_{1B} AR and β_2 AR.¹

In addition to their short-term metabolic effects, catecholamines also influence hepatocyte growth and differentiation: they increase hepatic DNA synthesis and are involved in the early phases of the regenerative response after hepatic injury or partial hepatectomy.² The mammalian liver displays unusual plasticity in that it can fully regenerate after extensive tissue loss or injury. This regenerative response is preceded by a temporary dedifferentiation of liver cells, characterized by the loss of liver-specific functions and gene products, such as the synthesis of albumin and transferrin, and the parallel rapid emergence of growth-related gene products, such as the protooncogenes *c-myc*, *c-jun*, *c-fos*, and *h-ras*.³ Adrenergic receptors also display a unique form of plasticity, best exemplified by studies of hepatic glycogenolysis in the rat. Although in the normal, adult, male rat this response is mediated exclusively by α_{1B} AR, after partial hepatectomy the same response is rapidly converted to a predominantly β_2 AR-mediated event.⁴ Interestingly, a similar conversion from α_1 - to β_2 -type response occurs in a number of other conditions, including glucocorticoid deficiency,⁵ hypothyroidism,⁶ toxin-induced liver regeneration,⁷ malignant transformation,⁸ cholestasis,⁴ fetal versus adult state,⁹ and dissociation of hepatocytes by enzymatic digestion.¹⁰⁻¹⁴ In many of these conditions, a corresponding decrease in the expression of α_{1B} AR and increase in the expression of β_2 AR have also been noted.^{4,6,12-17} Furthermore, the effects of other glycogenolytic hormones acting through calcium, such as vasopressin, or acting through cAMP, such as glucagon, were either unaffected or changed in a different direction than the corresponding AR response.¹¹ This strongly suggests that the conversion from α_{1B} - to β_2 -adrenergic control of glycogenolysis is related to corresponding inverse changes in the expression of the α_{1B} AR and β_2 AR genes,^{16,17} although it is clear that additional mechanisms, such as selective changes in the coupling of α_{1B} AR and β_2 AR to their respective G-proteins,^{1,10} or changes in G-protein expression¹⁸ are also involved. Because cellular dedifferentiation appears to be a common denominator among the

conditions associated with a switch from α_1 - to β_2 -adrenergic glycogenolysis, there may be common underlying mechanisms. Acute dissociation of liver cells by enzymatic digestion and partial hepatectomy both result in very rapid changes in the AR response^{4,10,11} and receptor gene expression,^{12-14,17} and they also share the pattern of altered expression of other affected genes, such as various protooncogenes.^{3,19} We used these two models in further studies to explore the underlying mechanisms.

MECHANISMS INVOLVED IN THE RAPID CONVERSION FROM α_1 - TO β_2 -ADRENERGIC GLYCOGENOLYSIS IN ACUTELY DISSOCIATED HEPATOCYTES

Role of Cyclooxygenase Products

Hepatocytes were isolated from adult, male rats by a collagenase perfusion protocol and were maintained in serum-free Krebs buffer containing 1.5% gelatin for improved viability. FIGURE 1 illustrates the typical α_1 -adrenergic response pattern of freshly isolated cells and β_2 -adrenergic response pattern of cells preincubated for 4 h, by the time-dependent decrease in the effectiveness of the α_1 -agonist, phenylephrine, and the parallel emergence of an effect of the β -agonist, isoproterenol, on phosphorylase *a* activity. The effects of dibutyl cAMP and of the calcium ionophore, A23187, were unaffected, indicating that the inverse changes in the α_1 - and β -adrenergic response must have occurred before the generation of the second messengers cAMP and calcium, respectively. Whereas prolonged *in vitro* incubation of hepatocytes was shown to be associated with up-regulation of β_2 AR and down-regulation of α_1 AR,¹²⁻¹⁴ the altered adrenergic activation of phosphorylase develops faster than the corresponding changes in receptor density. This suggests that, at least initially, the altered response must be due to inverse changes in the coupling of receptors to their respective post-receptor pathways.

Glucocorticoids are thought to produce most of their biological effects by inhibition of the breakdown of membrane phospholipids via phospholipase A_2 (PLA₂) and the subsequent generation of arachidonic acid (AA) metabolites, predominantly through the cyclooxygenase pathway. Inasmuch as glucocorticoid deficiency is one of the conditions associated with a conversion from α_1 - to β_2 -adrenergic glycogenolysis in rat liver,⁵ we hypothesized that increased activity of the PLA₂/cyclooxygenase system is a common pathway involved in the α/β change induced by various stimuli, including the acute dissociation of hepatocytes. Evidence in support of this hypothesis was provided by experiments using a "lipid trap" paradigm.²⁰ Defatted bovine serum albumin (BSA), which avidly binds fatty acids, can be used to trap fatty acids released by cells, whereas regular BSA, which is saturated with fatty acids, has no such effect. Hepatocytes incubated in their regular Krebs medium, or in medium in which 0.5% gelatin was replaced with 0.5% regular BSA, displayed a similar switch from α_1 - to β -adrenergic glycogenolysis after *in vitro* incubation for 4 h.²⁰ However, when the medium contained 0.5% defatted BSA, the cells that had been incubated for 4 h retained the α_1 -adrenergic response pattern observed in the freshly isolated cells.²⁰ Because the predominant fatty acid in the sn-2 position of membrane phospholipids is AA, we tested the effect of exogenous AA on the adrenergic activation of liver glycogen phosphorylase. A 20-min exposure of freshly isolated hepatocytes to 10 μ M AA, but not to stearic or palmitic acids, caused an acute shift in the receptor response from α_1 - to mixed α_1/β -type, and this change could be prevented by simultaneous exposure of the cells to the cyclooxygenase inhibitor, ibuprofen, but not the lipoxigenase inhibitor, nordihydroguaiaretic acid.²⁰

Ibuprofen also prevented the time-dependent shift of the receptor response from α_1 - to β -type.²⁰ Incubation of hepatocytes for 4 h with actinomycin D, which blocks the time-dependent conversion of the AR response, does not prevent the similar but more acute change caused by exogenous AA.²⁰ The rapid onset of the effects of exogenous AA and its independence from mRNA synthesis suggest that it is the coupling of AR rather than their expression that may be regulated by an AA metabolite. The more pronounced effects of AA and ibuprofen on the β - than on the α_1 -adrenergic response^{1,20} suggest that the primary target is the β_2 AR system. In agreement with this possibility, we found that hepatocytes from rats raised on an essential fatty acid (EFA)-free diet, which have markedly reduced AA and linoleic acid contents, fail to develop a β -adrenergic response on prolonged *in vitro* incubation.²¹

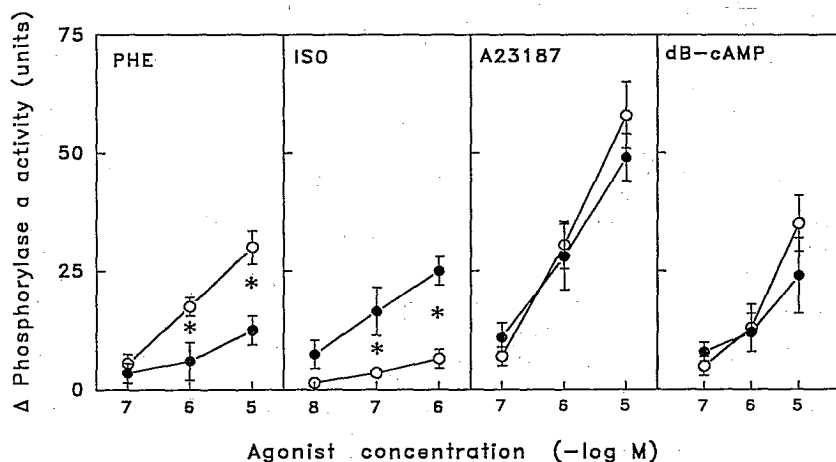


FIGURE 1. The effect of prolonged (4 h) incubation of isolated rat hepatocytes on the glycogenolytic response to various agonists. Phosphorylase *a* activity was determined⁶ in aliquots of freshly isolated cells (open circles) or cells preincubated for 4 h (filled circles) in the absence of drugs or after a 3-min exposure to the indicated concentrations of phenylephrine (PHE), isoproterenol (ISO), the calcium ionophore A23187, or dibutyl cAMP (dB-cAMP). Means \pm SE from five experiments are shown. Asterisk indicates significant difference between corresponding 0-h and 4-h values ($p < 0.05$). Baseline phosphorylase *a* activity was 20–25 units.⁶

Role of Protein Kinase C

Protein kinase C (PKC) plays a key role in signal transduction as well as in cell proliferation.²² Activation of PKC by phorbol esters inhibits differentiation and promotes growth in various tissues, including the liver where regeneration after partial hepatectomy is associated with activation of PKC.²³ Acute exposure of rat hepatocytes was also shown to selectively inhibit α_1 -receptor-mediated glycogenolysis,^{24,25} probably due to phosphorylation of the α_{1B} AR,²⁵ and in certain cell types phorbol esters potentiate β AR-mediated cAMP accumulation.^{26,27} These findings

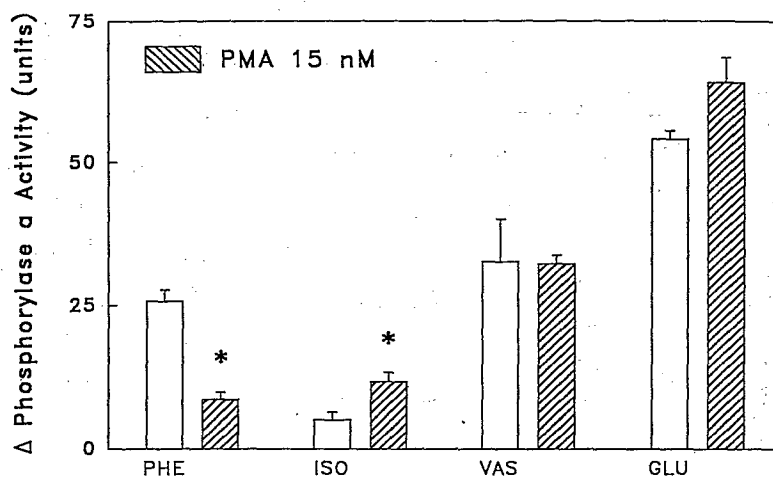


FIGURE 2. The effect of a phorbol ester (PMA) on the activation of phosphorylase by various glycogenolytic agents in freshly isolated hepatocytes. Cells were incubated for 10 min with vehicle (*open columns*) or 15 nM PMA (*cross-hatched columns*), and then exposed to vehicle (baseline) or to maximally effective concentrations of phenylephrine (10 μ M), isoproterenol (1 μ M), vasopressin (10 nM) or glucagon (10 nM). Means \pm SE from six experiments are shown.

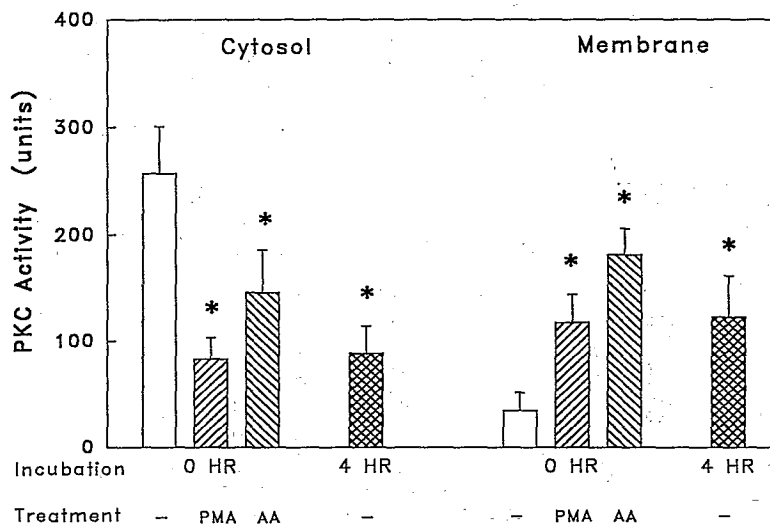


FIGURE 3. Drug-induced or time-dependent translocation of protein kinase C (PKC) activity in isolated hepatocytes. PKC activity was determined²⁹ in cytosol and plasma membrane fractions prepared from freshly isolated cells (0 h) or cells preincubated for 4 h. Zero-hour cells were exposed for 10 min to vehicle only, 15 nM PMA or 10 μ M AA before preparation of the subcellular fractions. Four-hour cells were exposed to vehicle only. Asterisk indicates significant difference from corresponding value in untreated 0-h cells ($p < 0.05$). Columns and bars represent means \pm SE ($n = 4$).

could suggest that activation of PKC is also involved in the conversion of the adrenergic activation of phosphorylase from an α_1 AR- to a β_2 AR-mediated event. The following observations support this possibility.

Effects of phorbol esters. Exposure of freshly isolated hepatocytes to 5 nM of phorbol 12-myristate, 13-acetate (PMA) for 4 min resulted in a marked reduction in the effect of phenylephrine, and a small but significant increase in the effect of isoproterenol on phosphorylase *a* activity, with no change in the effects of vasopressin or glucagon (FIG. 2). Exposure to phorbol 12-monoacetate, which does not activate PKC, had no effect on any of these drug responses. Experiments with different concentrations of PMA indicated that the EC_{50} of PMA for both the increase in the isoproterenol response and the decrease in the phenylephrine response was 3 nM, which is close to the K_d of PMA for PKC.²⁸

Time-dependent activation of PKC in isolated hepatocytes. It is well established that activation of PKC in various cell types is associated with its translocation from the cytosol to the membrane. We quantified PKC activity in cytosol and membrane

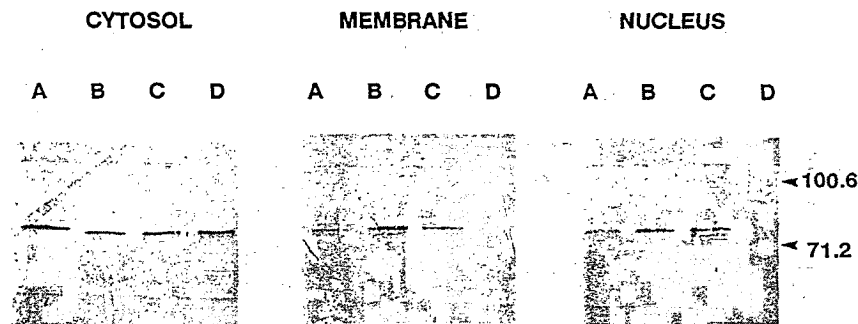


FIGURE 4. Translocation of PKC from cytosol to membrane and nucleus induced by PMA or by 4-h incubation. Detergent-solubilized subcellular fractions were prepared from cells pretreated as indicated, and were size-fractionated by electrophoresis on a 8% polyacrylamide gel, transferred to nitrocellulose, and blotted with a nonsubtype selective polyclonal antibody against PKC (UBI). Bands were visualized by biotinylated streptavidin. Band A, 0-h control; B, 0 h + PMA; C, 4-h control; D, 4 h + calphostin. For further explanation, see text.

fractions prepared from isolated hepatocytes, by measuring histone phosphorylation in the presence and absence of added phospholipids.²⁹ As illustrated in FIG. 3, in freshly isolated cells most of the activity was in the cytosol and very little in the membrane fraction. A 20-min exposure of these cells to 15 nM PMA resulted in a significant decrease in PKC activity in the cytosolic fraction and an increase in the membrane fraction, and a similar translocation occurred in cells incubated for 4 h in the absence of PMA. Interestingly, a similar cytosol-to-membrane translocation of PKC was induced by exposure of freshly isolated hepatocytes for 20 min to 10 μ M AA (FIG. 3). In these experiments, the increase in PKC activity in the membrane fraction was less than the decrease in the cytosolic fraction, suggesting that translocation may occur to additional sites, such as the nucleus. We examined the presence of immunoreactive PKC in cytosolic, membrane, and nuclear fractions by Western blotting, using a nonisoform selective polyclonal antibody against PKC. FIGURE 4 illustrates the results of an experiment with subcellular fractions prepared from freshly isolated hepatocytes exposed for 20 min to vehicle (A lanes) or to 15 nM

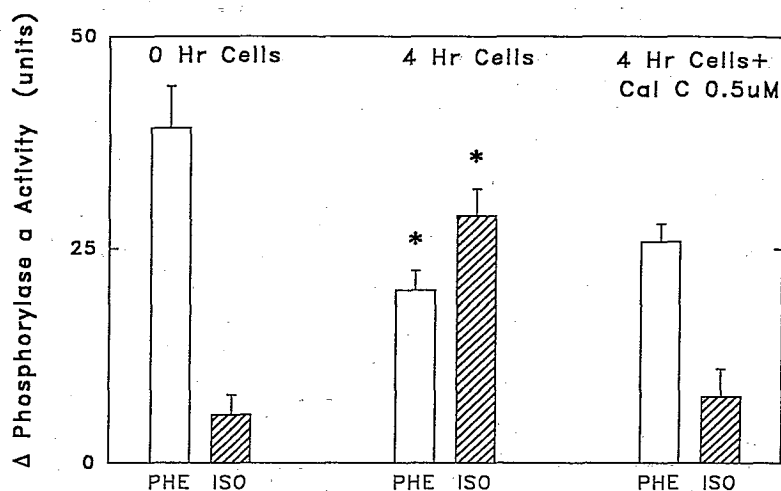


FIGURE 5. Calphostin C (Cal C) prevents the time-dependent conversion from α_1 - to β_2 -adrenergic glycogenolysis in isolated hepatocytes. Columns and bars (means + SE, $n = 5$) represent the increase in phosphorylase a activity caused by 10 μ M phenylephrine or 1 μ M isoproterenol under the indicated conditions.

PMA (B lanes), and from cells preincubated for 4 h with no drug added (C lanes) or incubated in the presence of 0.5 μ M calphostin C, a potent and selective inhibitor of PKC (D lanes). As seen in FIGURE 4, exposure of freshly isolated cells to PMA, or their *in vitro* incubation for 4 h without PMA, caused similar decreases in PKC in the cytosolic fraction and increases in both the membrane and nuclear fractions. The presence of calphostin throughout the 4-h incubation prevented the translocation of PKC to either membrane or nucleus.

Effects of calphostin C. Calphostin C not only prevented the time-dependent translocation of PKC, as evidenced by Western blots (see FIG. 4), but also prevented the parallel decrease in the α -adrenergic and increase in the β -adrenergic activation of phosphorylase (FIG. 5).

Together these observations strongly suggest that loss of cell-to-cell contact in the liver leads to activation of PKC, resulting in its translocation to both the plasma membrane and the nucleus, which is involved in the conversion from α_1 - to β_2 -adrenergic glycogenolysis.

TRANSCRIPTIONAL REGULATION OF HEPATIC α_{1B} -ADRENERGIC RECEPTORS

The rapidity with which changes in the adrenergic receptor response develop upon activation of PKC or of the AA pathway suggests that these changes occur at the level of the coupling of the receptors to their respective signal transduction pathways. However, primary culturing of hepatocytes also leads to corresponding, although more slowly developing, inverse changes in the expression of α_1 AR and β_2 AR.¹²⁻¹⁴ In order to obtain a more direct indicator of receptor synthesis, we

quantified the steady-state levels of α_{1B} AR and β_2 AR mRNAs in total RNA extracted from 0-h and 4-h cells. Of the three α_1 AR subtypes cloned to date, only the α_{1B} AR has been found to be expressed in the rat liver.³⁰ mRNA levels were measured by highly sensitive DNA excess solution hybridization assays with sensitivity limits in the range of 0.1 amol mRNA/ μ g total RNA.^{16,17} A progressive decrease in α_{1B} AR mRNA and a parallel increase in β_2 AR mRNA were detected which reached statistical significance by the second to third hour of incubation.¹⁷ FIGURE 6 illustrates a similar inverse change, that is, a decrease in α_{1B} AR mRNA and an increase in β_2 AR mRNA induced by partial hepatectomy, as detected in a Northern blot of poly A⁺ RNA prepared from the livers of sham-operated animals, or from the residual liver tissue within 2 or 6 h of 2/3 partial hepatectomy. The α_{1B} AR and β_2 AR mRNAs were detected in mRNA preparations obtained from the same livers. FIGURE 6 also illustrates the well-documented mRNA heterogeneity for both receptors. The α_{1B} AR has three mRNAs: a major 2.7 kb and two minor bands at 3.3

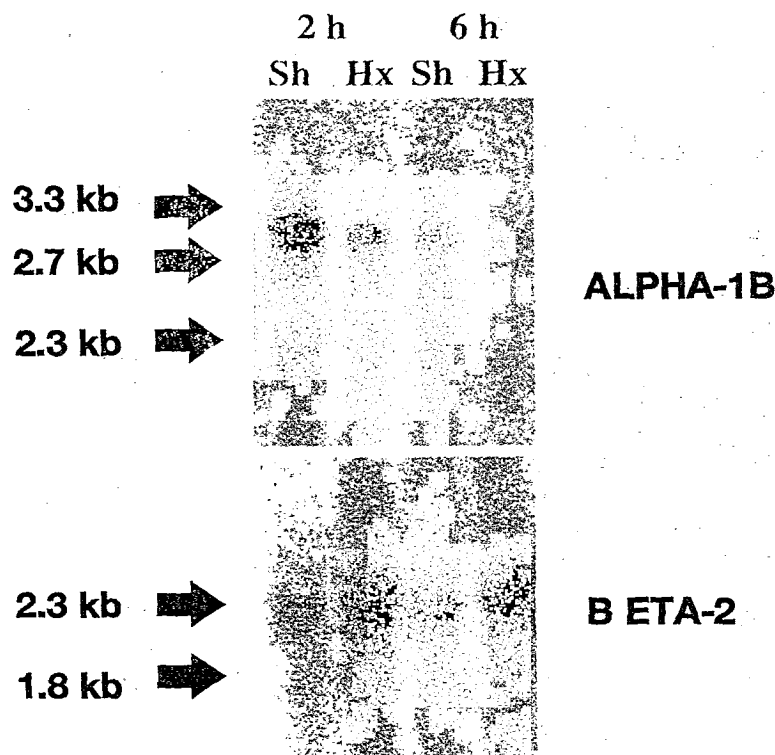


FIGURE 6. Reciprocal changes in the steady-state levels of α_{1B} AR and β_2 AR mRNAs induced by partial hepatectomy. Northern blots of poly A⁺ RNA prepared from the liver of sham-operated (Sh) or 2/3 hepatectomized (Hx) male Sprague-Dawley rats (200 g) 2 or 6 h following surgery were hybridized with [³⁵S]labeled cDNA probes for α_{1B} AR and β_2 AR. Bands were visualized by a phosphorimager. Note the Hx-induced opposite changes in the α_{1B} AR and β_2 AR message in the same RNA preparations.

and 2.3 kb,³¹ of which the 3.3 kb species is only expressed in liver.³² Nuclear run-on assays indicate that these inverse changes in steady-state mRNA levels can be accounted for by a corresponding increase or decrease in the rate of transcription of the β_2 AR or α_{1B} AR genes, respectively (not shown).

Once it is clear that a major mechanism by which receptor expression is regulated is at the level of the transcription of the receptor gene, it is apparent that further analysis requires a detailed characterization of the regulatory domains of the gene and of the *trans*-acting factors that interact with these domains to direct or to modulate the rate of transcription. As a first step towards this goal, we isolated and sequenced the gene encoding the rat α_{1B} AR and characterized its 5'-flanking region.³³ Unlike the intronless genes encoding other adrenergic receptors cloned to date, the rat α_{1B} AR has a large intron (> 16 kb) interrupting its coding region. A similar feature is evident in the human α_{1B} AR gene, published at the time this work was completed.³⁴ Analysis of the sequence of the first 1000 bp immediately upstream from the coding region indicates the absence of TATA and CCAAT boxes and G + C rich regions, features characteristic of housekeeping genes.³³ The 5'-flanking region also contains consensus sequences that are recognized response elements for various *trans*-acting factors, such as AP1, cAMP (CRE), glucocorticoid receptor (GRE), and thyroid hormone receptor (TRE),³³ which are probably involved in the well-documented regulation of α_{1B} AR by PKC,³⁵ cAMP,³⁶ corticosteroids,³⁷ and thyroid hormones,^{6,15,16} respectively. Primer extension analyses using 5' upstream primers identified transcription start points at -54 and -57 bp (tsp1), -443 bp (tsp2), and a cluster between -1035 and -1340 bp (tsp3).³¹ Further analysis by transient transfections of putative promoter/CAT constructs revealed that the α_{1B} AR gene has three independent promoters, as illustrated in FIGURE 7.³¹ The promoters are located at -49 to -127 bp (P1), -432 to -813 bp (P2), and -1107 to -1363 bp (P3) upstream from the start codon, and they direct transcription from tsp1, tsp2, and tsp3, to generate mRNA species of 2.3, 2.7, and 3.3 kb in length, respectively.³¹ Analysis of the structure of these promoters indicate that P1 and P2 have features of housekeeping-like promoters, whereas P3, which is responsible for the liver-specific expression of the 3.3 kb mRNA species,³² has both a TATA and a CCAAT box and is flanked by recognition sites for liver specific transcription factors, such as the CCAAT/enhancer binding protein and hepatocyte nuclear factor-5.³¹ These findings suggest that differential control of these promoters may underlie the well-documented developmental and tissue-specific regulation of the α_{1B} AR. Studies are in progress to identify the regulatory domains of the β_2 AR and the *trans*-acting factors that bind to these domains to control transcription of the β_2 AR gene. If liver-specific factors are found that are involved in the transcriptional regulation of both the α_{1B} AR and the β_2 AR, the possible role of such factors in the inverse regulation of these two receptors will be further explored.

POSSIBLE BIOLOGICAL SIGNIFICANCE

Inverse regulation of α_{1B} AR and β_2 AR has been observed in a number of different physiological and pathological conditions (see introduction). The remarkable similarity of the altered receptor response pattern under these various conditions suggests that it represents a unique form of receptor regulation. Furthermore, the close parallel between conversion of the AR response and a shift from liver-

specific to growth-related functions suggests a role in the process of liver cell growth and differentiation. Whereas the activation of glycogenolysis by α_{1B} AR or β_2 AR is a unidirectional response, this is not the case for effects on liver cell proliferation. α_{1B} AR are mitogenic, increase DNA synthesis, and their inhibition can prevent liver

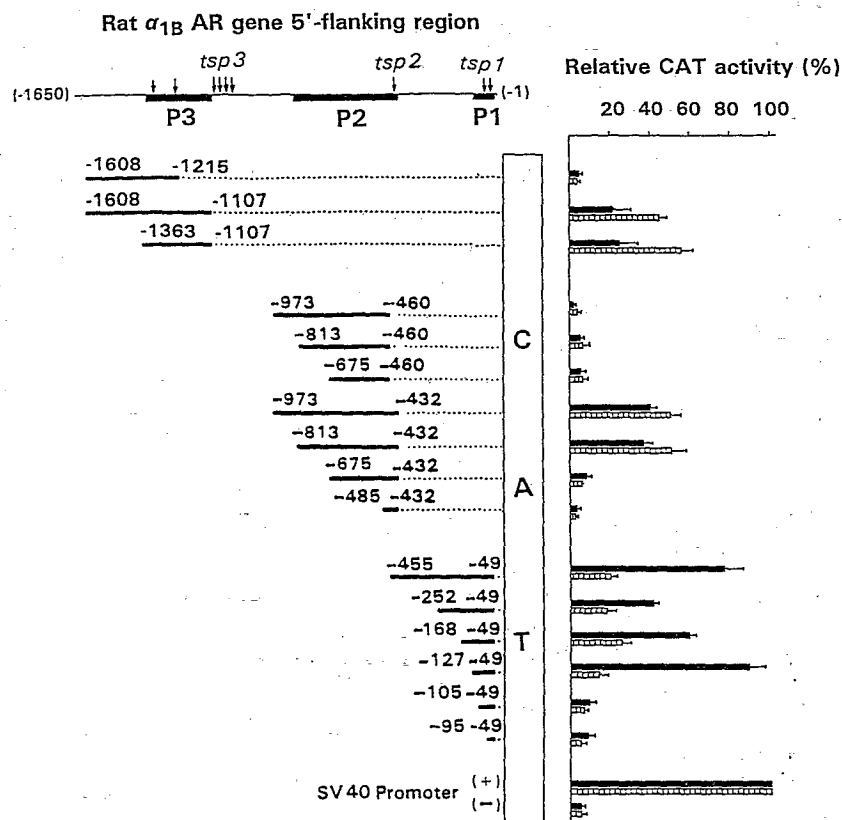


FIGURE 7. Characterization of three promoters of the α_{1B} AR receptor gene. The left side is a schematic representation of the pCAT constructs used in cell transfection experiments; the right side shows CAT activity in Hep3B (solid bars) and in DDT₁ MF-2 cells (cross-hatched bars), expressed as % of positive control. CAT activities (means \pm SE, $n = 3$) are corrected for transfection efficiency, as described.³¹ The positions of the three promoters (horizontal bars) and 3 tsp (arrows) are indicated on the line representing the 5'-flanking region. (From Gao and Kunos.³¹ Reproduced, with permission, from the *Journal of Biological Chemistry*.)

regeneration after partial hepatectomy.² In contrast, in proliferating liver cells, stimulation of β_2 AR strongly inhibits the G1-S transition,³⁸ and thus may be involved in the termination of the proliferative process. This, and the reported shift from α_1 AR to β_2 AR in human hepatocellular carcinoma,³⁹ could suggest that this form of

regulation is important in terminating hepatocyte proliferation in order to prevent its potential progression into malignant transformation.

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